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Impact of Plant Density and Mepiquat Chloride on Growth, Yield, and Silymarin Content of *Silybum marianum* Grown under Mediterranean Semi-Arid Conditions

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Abstract: Milk thistle (*Silybum marianum* (L.) Gaertn.) is a promising new crop in the Mediterranean region. Its seeds contain silymarin, a complex of flavonolignans, which is widely used in the pharmaceutical industry, mainly to produce dietary supplements. To meet the increasing demand for milk thistle, the production and productivity of milk thistle should also be optimized by employing adequate cultivation practices. In the present study, a two-year field experiment was conducted to assess the effects of plant density and a plant growth regulator on milk thistle crop growth, seed yield, and silymarin accumulation under Mediterranean semi-arid conditions. Our results showed that plant density had a significant impact on milk thistle crop growth and seed yield. The main crop characteristics, such as height, aboveground biomass, and seed yield were greatest when plant density was the highest. Increased plant density significantly reduced the silymarin content only in 2018. In contrast, mepiquat chloride (MC) treatment did not affect the following traits: plant biomass, relative chlorophyll content, silymarin content, and production. Nevertheless, mepiquat chloride reduced the plant height by 7.9–14.8%, depending on the application rates and growth conditions. Moreover, the impact of climatic conditions on milk thistle production and quality was significant, since the lowest values of silymarin content and seed yield were recorded in the year with drought conditions during the period from March to May.

Keywords: biomass; flavonolignans; Mediterranean environment; milk thistle; plant growth regulators

1. Introduction

Milk thistle (*Silybum marianum* (L.) Gaertn.) is a broadleaved species of the family Asteraceae that is cultivated in several regions worldwide and is one of the most widely used medicinal plants [1]. Currently, it is a very popular herbal remedy used by patients suffering from liver diseases [2,3], and dietary supplements containing silymarin are among the best-selling plant-derived pharmaceuticals in Europe and the USA [4,5]. Silymarin is contained in the extract derived from the seeds of milk thistle and is a complex mixture of at least six flavonolignans (silybin A and B, isosilybin A and B, silychristin, and silydianin) and one flavonoid (taxifolin) [6,7]. The content of silymarin in seeds can reach up to 7.71% of the seed dry weight [8]. Besides its hepatoprotective action against several liver diseases, silymarin exhibits anticancer activity, anti-inflammatory, antifibrotic and antioxidant properties [2,4,9–11].

Milk thistle yield and quality (i.e., silymarin content) is dependent on the environmental conditions, soil properties, fertilization, plant density, harvest stage, and genotype [1,8,12,13]. Among these agronomic parameters, the selection of appropriate plant density is critical in order to optimize growth and yield. To our knowledge, the effects of plant density on milk thistle yield and silymarin content has not been extensively studied under Mediterranean semi-arid conditions. In Turkey, according to Katar et al. [13] a high crop density (25 × 10 cm) increased both the seed and silymarin yield; however, achieving the optimum crop density is also dependent on environmental conditions [14]. Under Mediterranean conditions, overpopulations may increase the risk of drought stress in this crop during its reproductive stages. In this context, it is important to examine the impacts of high plant density on the growth and seed yield of milk thistle under semi-arid conditions. In a recent study, Zangani et al. [15] reported that the seed yield of milk thistle was significantly reduced under water stress conditions. In contrast, the biosynthesis of silymarin in milk thistle seeds was also found to be enhanced under drought stress conditions [16].

In addition, seed dispersal is a major challenge faced by milk thistle producers and is the key limiting factor in economically viable milk thistle production. This problem is aggravated when harvesting is conducted using mechanical methods. In this context, it is important to develop new cultivars with low seed dispersal. According to Martinelli [17], the implementation of a mutagenesis program for this species may help identify useful traits to reduce seed dispersal when the plants reach maturity. Moreover, to minimize the yield losses during the mechanical harvest of this crop, the plant height should be reduced, because milk thistle can grow to be 200 cm tall. The application of plant growth regulators, such as mepiquat chloride may help achieve these goals as several plant species respond to the application of plant growth retardants. Nichols et al. [18] observed that mepiquat can be a useful management tool to: (a) retard the growth of cotton and (b) improve the harvest efficiency of fields that normally require plant height control. Similarly, Suzuki et al. [19] reported that mepiquat chloride and paclobutrazol significantly reduced the height of sunflower plants due to the inhibition of gibberellin biosynthesis. Other studies show that milk thistle plants also respond to the application of plant growth regulators [20,21]. Among the effects of these regulators, such as mepiquat chloride and thidiazuron, were the increase of milk thistle seed yield and silymarin accumulation [20,21]. Moreover, the reduction of plant height is necessary for pest and disease control on milk thistle crop.

Taking this background into consideration, this study aimed to determine the effects of plant density and mepiquat chloride on growth, seed yield, and silymarin content in milk thistle grown under semi-arid conditions. Height, dry weight, relative chlorophyll content, number of flower heads, 1000-seed weight, silymarin content, and seed yield were the main parameters used to examine the effects of plant density and mepiquat chloride on milk thistle performance.

2. Materials and Methods

2.1. Experimental Site and Design

A two-year field study was conducted at the experimental farm of the University of Thessaly in Velestino (22°75′67″ E, 39°39′60″ N), located in the prefecture of Thessaly, in 2015–2016 and 2017–2018. The soil texture was sandy clay loam (clay (26%), silt (36%), and sand (38%)), with a pH of 7.4. The mean monthly temperatures and volume of precipitation throughout the experimental periods, from November to June, are presented in Figure 1. The total precipitation during these experimental periods was 259 mm in 2015–2016 and 481 mm in 2017–2018.

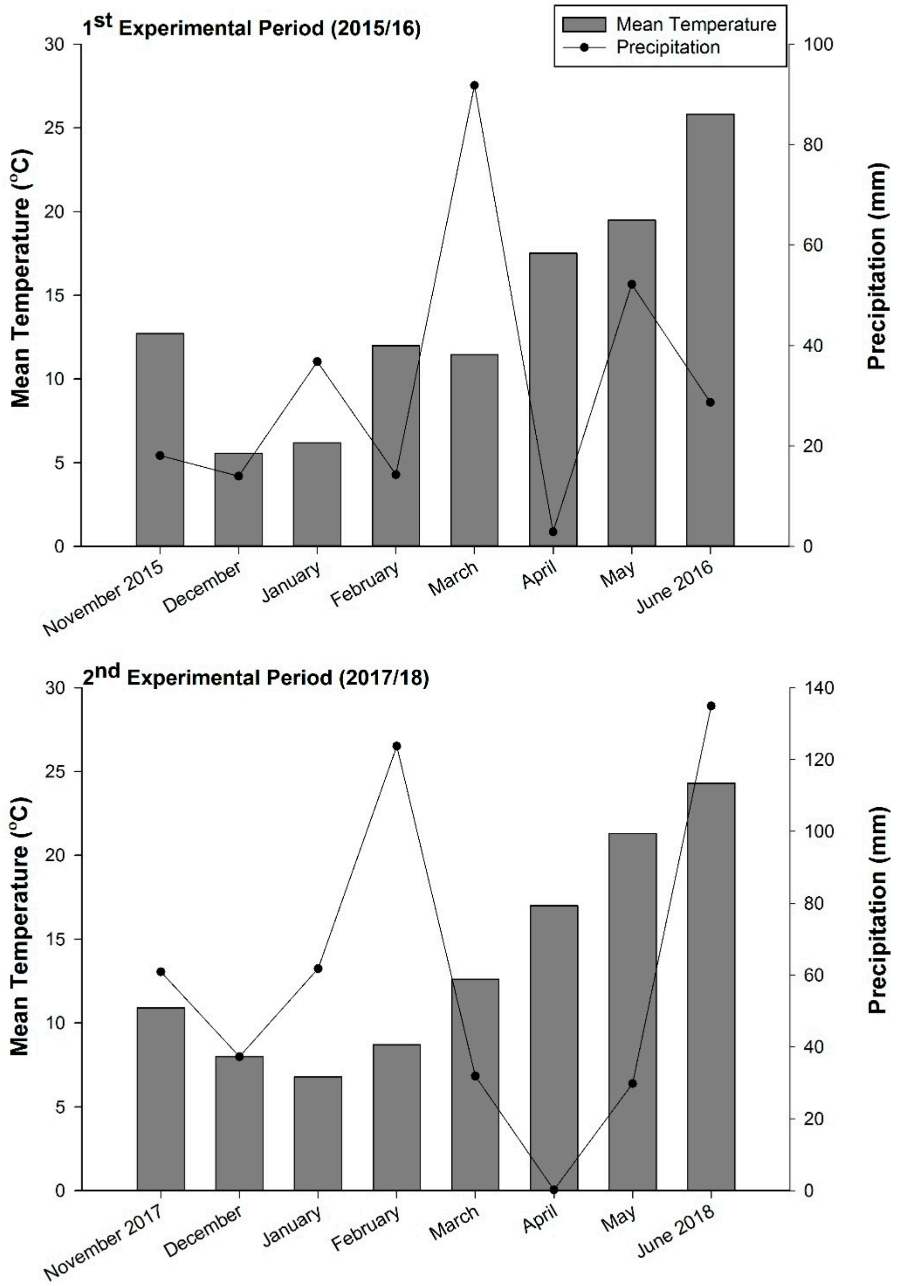

Figure 1. Mean temperature and monthly precipitation during the two experimental periods (November 2015–June 2016 and November 2017–June 2018, respectively) at Velestino in central Greece.

A local population ('Agios Georgios') of milk thistle, originating from central Greece, was hand sown on 5 November 2015 and 30 October 2017, for the first and second growing seasons, respectively. The field experiment was split-plot design with three replications. Plant density was the main plot factor; density A was 28 plants m^{-2} (50 cm × 7 cm) and density B was 40 plants m^{-2} (35 cm × 7 cm). The mepiquat chloride (MC) (Pix 5 SL, BASF, Athens, Greece) was the sub-plot factor, with plot sizes of 10 m^2 (2 m × 5 m); the MC treatments were: (1) untreated control, (2) mepiquat chloride at the rate of 75 g a.i. ha^{-1} (MC 75), (3) mepiquat chloride at the rate of 100 g a.i. ha^{-1} (MC 100), and (4) mepiquat chloride applied twice, 2 weeks apart, at the rates of 37.5 g a.i. ha^{-1} and 75 g a.i. ha^{-1} for the first and second applications, respectively (MC 112). Thinning of milk thistle seedlings was done at the four leaf stage to achieve the desired density in row for each treatment. Mepiquat chloride was applied during the rapid and excessive vegetative growth stage when the milk thistle plants were about 40 cm tall and it was combined with the surfactant alkylethersulfate sodium salt (Biopower SL, Bayer, Athens, Greece) at the rate of 380 g a.i. ha^{-1}. MC application was done with a hand-held plot sprayer, at a pressure of 300 kPa, using hollow cone nozzles and a total water volume of 500 L ha^{-1}. This high spray volume results in improved coverage of crop canopy. Weed management in the plots was carried out by hand hoeing in the middle of February.

2.2. Sampling, Measurements and Methods

2.2.1. Measurements during Crop Growth

Rosette diameter, height, and chlorophyll content were measured from ten plants randomly selected from each sub-plot, avoiding plants at the beginning and the end of the rows. The rosette diameter measurements were made before the application of mepiquat chloride which was applied during the stem elongation period, while the relative chlorophyll content (SPAD values) was estimated at the phenological growth stages 53–54 and 63–64 of the BBCH scale described by Martinelli et al. [22], using the SPAD-502 chlorophyll content meter (Konica Minolta Optics, Osaka, Japan). For the measurement of aboveground dry biomass, five subsequent plants in the interior rows from each plot were selected. The biomass was determined after drying at 60 °C for 96 h.

2.2.2. Measurements at Harvest

The number of flower heads (capitulum) were recorded for ten subsequent plants in the interior rows selected from each plot, avoiding plants at the beginning and the end of the rows. To determine seed yield during the ripening stage, the flower heads from ten plants were hand harvested from the central sowing row. In order to reduce seed dispersal, the harvest was performed when at least 60% of the heads had reached the phenological growth stage 88 of the BBCH scale. After the harvest, the 1000-seed weight was calculated by randomly weighing 3 × 100 seeds from each sub-plot.

2.2.3. Oil and Silymarin Content Determination

Initially, 5 g of dried seeds were ground using a laboratory grinder and then the oil was extracted with 200 mL of hexane by a Soxhlet apparatus for 4 h. The oil was recovered by evaporating the solvent to dryness on a rotary evaporator at 40 °C. The silymarin constituents were subsequently extracted from the seed sample with 200 mL of methanol, in a Soxhlet apparatus for 4 h. The extract was evaporated to dryness under vacuum at 40 °C and reconstituted in 25 ml of high performance liquid chromatography (HPLC) grade methanol. The reconstituted extract (1 ml) was diluted with methanol to 25 ml, and then used for silymarin determination.

Silymarin analysis was performed with an HPLC system (HP 1100 Liquid Chromatograph, Hewlett-Packard GmbH, Waldbronn, Germany) coupled to a ternary-delivery system and a variable wavelength UV detector. Chromatographic separation of silymarin compounds was achieved using a Reprosil Gold C-18 column (5.0 µm, 250 × 4.6 mm), operating at 40 °C, while a sample of 20 µL was injected. A solvent system of methanol and water containing formic acid (0.1%) was used. Gradient elution was performed with increasing amounts of methanol to water at a flow rate of 1 mL/min, as described in a previous study [8], while detection was made at 288 nm.

A silymarin standard (Sigma-Aldrich) dissolved in methanol was used for the determination of the retention time of each silymarin constituent (Figure 2). Calibration curves were prepared using the silybinin standard (Sigma-Aldrich, St. Louis, MS, USA) solutions in methanol. The content of each component of silymarin was calculated using their peak area and the calibration curve established for silybin A.

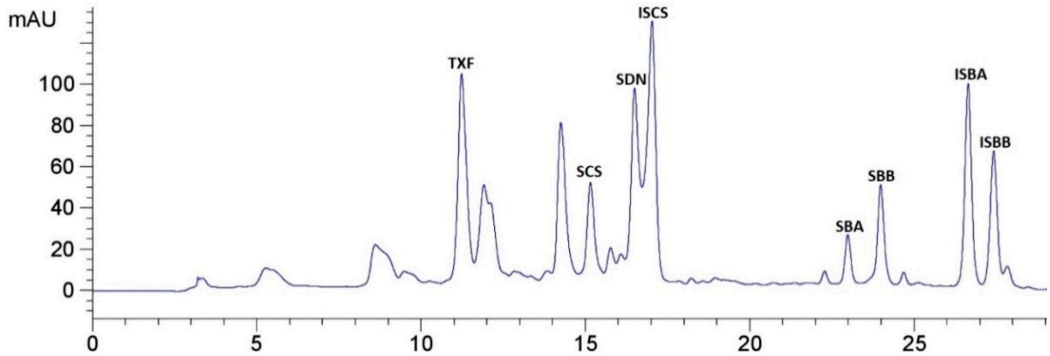

Figure 2. HPLC chromatogram of silymarin constituents of the milk thistle population 'Agios Georgios' originating from central Greece. TXF: Taxifolin, SCS: Silychristin, SDN: Silydianin, ISCS: Isosilychristin, SBA: Silybin A, SBB: Silybin B, ISBA: Isosilybin A, ISBB: Isosilybin B.

2.3. Statistical Analysis

A three-way analysis of variance (ANOVA) was applied to evaluate the main effects of plant density (Factor 1), mepiquat chloride treatments (Factor 2), and year (Factor 3) as well as the interactions between them. The statistical analysis was carried out using the SigmaPlot 12 Software (Systat Software, San Jose, CA, USA). For each experimental period, the means were compared using a Fisher's Least Significant Difference test (LSD, $p \leq 0.05$) when ANOVA (two-way analysis of variance) was significant at $p \leq 0.05$. Finally, a Pearson's correlation analysis was performed to evaluate the relationships between the milk thistle characteristics.

3. Results

3.1. Crop Parameters

Plant density had a significant effect on the crop parameters, such as plant height, aboveground biomass, and rosette diameter. The mean values of plant height were 183.3 cm in the first growing season and 144.9 cm in the second growing season (Table 1) for the final height measurements. In both growing seasons, the greatest values for plant height and rosette diameter (Figure 3) were recorded for plant density A. For plant height there were significant differences among the four mepiquat chloride treatments. The shortest plant heights were recorded with the MC 100 and MC 112 treatments, while there were no interactions between plant density and mepiquat chloride for plant height.

The dry weight of the milk thistle crop was also affected by plant density (Table 2), as the greatest values were recorded with the high-density crops. In contrast, mepiquat chloride did not affect the aboveground biomass of the milk thistle crop. The mean values of dry weight were 24,488 kg ha^{-1} and 20,299 kg ha^{-1}, in 2016 and 2018, respectively, for the final weight measurements. Moreover, the relative chlorophyll content (SPAD values) was reduced at the highest plant density, while no significant differences were observed among the MC treatments (Table 3). The mean SPAD values were 35.6 in the first growing season and 37.3 in the second growing season for the second measurement.

Table 1. Influence of plant density and mepiquat chloride (MC) treatments on the plant height of milk thistle at 8, 24, and 38 days after MC application in 2015–2016 and 2017–2018.

Treatments	Height (cm)					
	8 DAA[1]		24 DAA		38 DAA	
	2015/2016	2017/2018	2015/2016	2017/2018	2015/2016	2017/2018
Plant density (D)						
Density A	67.5 a	70.4 a	142.1 a	129.7 a	188.4 a	156.1 a
Density B	64.8 a	60.3 b	134.0 b	114.5 b	178.1 b	133.6 b
$LSD_{5\%}$	-	4.20	6.70	5.01	5.89	9.52
Mepiquat chloride (MC)						
Control	68.1 a	68.5 a	152.0 a	135.8 a	196.7 a	158.5 a
MC75	66.6 a	62.1 a	137.3 b	122.5 b	181.2 b	145.2 ab
MC100	64.1 a	64.1 a	131.2 b	114.2 c	175.6 b	140.8 bc
MC112	65.9 a	66.7 a	131.6 b	115.9 c	179.6 b	135.0 bc
$LSD_{5\%}$	-	-	9.48	7.09	8.34	13.46
	p-value from ANOVA					
D	0.051		<0.001		<0.001	
MC	0.766		<0.001		<0.001	
Year	0.788		<0.001		<0.001	
D × MC	0.990		0.288		0.104	
D × Year	0.249		0.082		0.027	
MC × Year	0.929		0.985		0.568	
D × MC × Year	0.989		0.836		0.192	

[1] DAA: Days after MC application, Density A: 28 plants m^{-2} and density B: 40 plants m^{-2}, MC 75: MC applied at the rate of 75 g a.i. ha^{-1}, MC 100: MC applied at the rate of 100 g a.i. ha^{-1}, and MC 112: MC applied twice, 2 weeks apart, at the rates of 37.5 g a.i. ha^{-1} and 75 g a.i. ha^{-1} for the first and second applications, respectively. Means followed by different letters within the same column indicate significant differences (for each factor) according to the Fisher's Least Significant Difference test ($p < 0.05$).

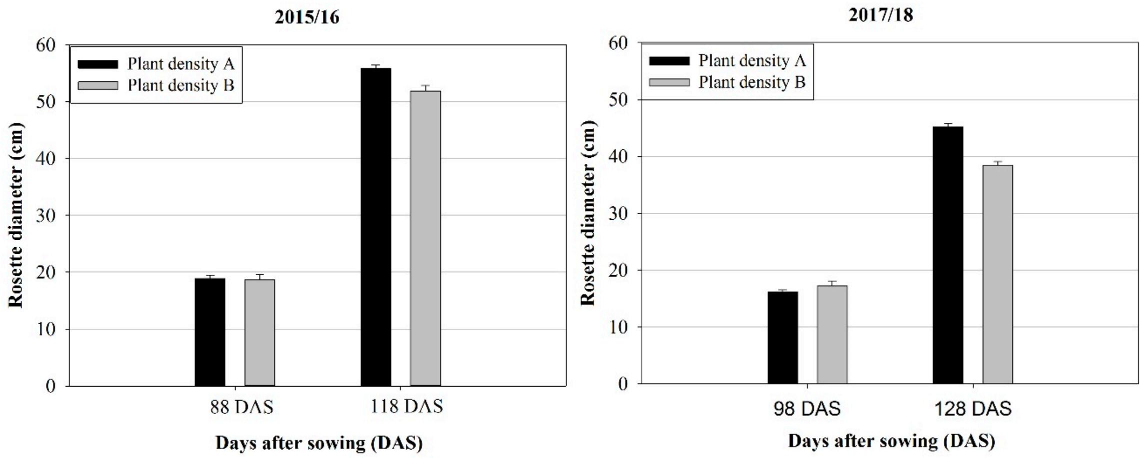

Figure 3. Influence of plant density (density **A**: 28 plants m^{-2} and density **B**: 40 plants m^{-2}) on rosette diameter (cm) of milk thistle crop at the first measurement (88 and 98 days after sowing (DAS) in 2015–2016 and 2017–2018, respectively) and second measurement (118 and 128 DAS in 2015–2016 and 2017–2018, respectively). Vertical bars indicate the standard errors of the means.

Table 2. Influence of plant density and mepiquat chloride (MC) treatments on dry weight (kg ha^{-1}) of milk thistle crops at 8, 24, and 38 days after MC application in 2015–2016 and 2017–2018.

Treatments	Dry Weight (kg ha^{-1})					
	8 DAA[1]		24 DAA		38 DAA	
	2015/2016	2017/2018	2015/2016	2017/2018	2015/2016	2017/2018
Plant density (D)						
Density A	10,199 b	9615 b	21,947 b	16,204 b	23,537 b	19,089 b
Density B	11,182 a	10,681 a	24,017 a	18,074 a	25,440 a	21,509 a
$LSD_{5\%}$	911	734	1,446	925	1497	1743
Mepiquat chloride (MC)						
Control	11,522 a	10,256 a	22,460 a	17,012 a	23,843 a	20,288 a
MC75	10,710 a	9982 a	23,235 a	17,060 a	25,062 a	19,647 a
MC100	10,020 a	10,173 a	22,853 a	16,941 a	24,950 a	20,489 a
MC112	10,510 a	10,180 a	23,380 a	17,544 a	24,099 a	20,772 a
LSD	-	-	-	-	-	-
	p-value from ANOVA					
D	<0.001		<0.001		<0.001	
MC	0.242		0.612		0.863	
Year	0.058		<0.001		<0.001	
D × MC	0.734		0.682		0.954	
D × Year	0.883		0.806		0.637	
MC × Year	0.330		0.937		0.518	
D × MC × Year	0.986		0.947		0.718	

[1] DAA: days after MC application, Density A: 28 plants m^{-2} and density B: 40 plants m^{-2}, MC 75: MC applied at the rate of 75 g a.i. ha^{-1}, MC100: MC applied at the rate of 100 g a.i. ha^{-1}, and MC 112: MC applied twice, 2 weeks apart, at the rates of 37.5 g a.i. ha^{-1} and 75 g a.i. ha^{-1} for the first and second applications, respectively. Means followed by different letters within the same column indicate significant differences (for each factor) according to the Fisher's Least Significant Difference test ($p < 0.05$).

Table 3. Effects of plant density and mepiquat chloride (MC) treatments on chlorophyll content (SPAD values) at 24 and 38 days after MC application in 2015/2016 and 2017/2018.

Treatments	Chlorophyll Content (SPAD values)			
	24 DAA[1]		38 DAA	
	2015/2016	2017/2018	2015/2016	2017/2018
Plant density (D)				
Density A	39.7 a	40.2 a	38.0 a	38.7 a
Density B	37.1 b	37.9 b	33.1 b	35.8 b
$LSD_{5\%}$	1.84	0.74	1.99	0.86
Mepiquat chloride (MC)				
Control	38.7 a	39.2 a	36.6 a	37.1 a
MC75	38.2 a	38.4 a	37.2 a	36.5 a
MC100	37.6 a	39.8 a	34.5 a	37.5 a
MC112	39.1 a	38.9 a	34.0 a	38.0 a
$LSD_{5\%}$	-	-	-	-
	p-value from ANOVA			
D	<0.001		<0.001	
MC	0.702		0.445	
Year	0.156		0.002	
D × MC	0.701		0.871	
D × Year	0.796		0.064	
MC × Year	0.345		0.009	
D × MC × Year	0.513		0.869	

[1] DAA: days after MC application, Density A: 28 plants m^{-2} and density B: 40 plants m^{-2}, MC 75: MC applied at the rate of 75 g a.i. ha^{-1}, MC100: MC applied at the rate of 100 g a.i. ha^{-1}, and MC 112: MC applied twice, 2 weeks apart, at the rates of 37.5 g a.i. ha^{-1} and 75 g a.i. ha^{-1} for the first and second applications, respectively. Means followed by different letters within the same column indicate significant differences (for each factor) according to the Fisher's Least Significant Difference test ($p < 0.05$).

3.2. Seed Yield and Components

The mean values of seed yield were 2075 kg ha^{-1}, in the first growing season and 1617 kg ha^{-1}, in the second growing season (Table 4). For seed yield, a significant ($p < 0.001$) plant density × year interaction was observed. In the first growing season the highest seed yield was observed with plant density B, while in the second growing season, the highest seed yield was recorded with plant density A. In the first growing season, plant density did not significantly affect the number of flower heads and the 1000-seed weight, while in the second growing season the largest number of flower heads was recorded with plant density A. There were no significant differences among the four MC treatments for flower head numbers, 1000-seed weight, and seed yield.

Table 4. Effects of plant density and mepiquat chloride treatments on the number of heads (No plant^{-1}), 1000-seed weight (g), and seed yield (kg ha^{-1}) in 2015–2016 and 2017–2018.

Treatments	Head Number (No plant^{-1})		1000-Seed Weight (g)		Seed Yield (kg ha^{-1})	
	2015/2016	2017/2018	2015/2016	2017/2018	2015/2016	2017/2018
Plant density (D)						
Density A	5.95 a	5.53 a	20.37 a	22.29 a	1929 b	1790 a
Density B	4.97 a	4.19 b	20.44 a	21.40 b	2222 a	1444 b
$LSD_{5\%}$	-	0.712	-	0.769	186	290
Mepiquat chloride (MC)						
Control	5.79 a	5.00 a	20.18 a	21.46 a	2058 a	1723 a
MC75	5.50 a	4.79 a	20.33 a	21.93 a	2074 a	1497 a
MC100	5.33 a	4.72 a	20.89 a	22.26 a	2075 a	1585 a
MC112	5.22 a	4.92 a	20.22 a	21.73 a	2090 a	1663 a
$LSD_{5\%}$	-	-	-	-	-	-
	p-value from ANOVA					
D	<0.001		0.158		0.742	
MC	0.808		0.279		0.792	
Year	0.045		<0.001		<0.001	
D × MC	0.900		0.414		0.817	
D × Year	0.539		0.094		<0.001	
MC × Year	0.939		0.980		0.758	
D × MC × Year	0.885		0.856		0.941	

Density A: 28 plants m^{-2} and density B: 40 plants m^{-2}, MC 75: MC applied at the rate of 75 g a.i. ha^{-1}, MC100: MC applied at the rate of 100 g a.i. ha^{-1}, and MC 112: MC applied twice, 2 weeks apart, at the rates of 37.5 g a.i. ha^{-1} and 75 g a.i. ha^{-1} for the first and second applications, respectively. Means followed by different letters within the same column indicate significant differences (for each factor) according to the Fisher's Least Significant Difference test ($p < 0.05$).

3.3. Oil Content and Yield

The mean values of oil content were 26.8% in the first growing season and 24.7% in the second growing season, without any significant effects of plant density and mepiquat chloride treatments (Table 5). Moreover, mepiquat chloride alone did not affect the oil yield. For oil yield, a significant ($p < 0.001$) plant density × year interaction was observed. In the first growing season, the highest oil yield (591 kg ha^{-1}) was observed with plant density B, while in the second growing season, the highest oil yield (444 kg ha^{-1}) was recorded with plant density A.

Table 5. Effects of plant density and mepiquat chloride treatments on oil content (%) and yield (L ha^{-1}) in 2015/2016 and 2017/2018.

Treatments	Oil Content (%)		Oil Yield (kg ha^{-1})	
	2015/2016	**2017/2018**	**2015/2016**	**2017/2018**
Plant density (D)				
Density A	27.0 a	24.8 a	521 b	444 a
Density B	26.6 a	24.5 a	591 a	353 b
$LSD_{5\%}$	-	-	52.48	71.26
Mepiquat chloride (MC)				
Control	26.7 a	25.0 a	550 a	428 a
MC75	26.4 a	24.2 a	548 a	362 a
MC100	27.3 a	24.8 a	566 a	392 a
MC112	26.9 a	24.6 a	560 a	412 a
$LSD_{5\%}$	-	-	-	-
	p-value from ANOVA			
D	0.268		0.805	
MC	0.373		0.657	
Year	<0.001		<0.001	
D × MC	0.119		0.964	
D × Year	0.917		<0.001	
MC × Year	0.812		0.716	
D × MC × Year	0.819		0.920	

Density A: 28 plants m^{-2} and density B: 40 plants m^{-2}, MC 75: MC applied at the rate of 75 g a.i. ha^{-1}, MC 100: MC applied at the rate of 100 g a.i. ha^{-1}, and MC 112: MC applied twice, 2 weeks apart, at the rates of 37.5 g a.i. ha^{-1} and 75 g a.i. ha^{-1} for the first and second applications, respectively. Means followed by different letters within the same column indicate significant differences (for each factor) according to the Fisher's Least Significant Difference test ($p < 0.05$).

3.4. Silymarin Content and Yield

Regarding the silymarin content, there were no significant differences between the MC treatments, while the highest silymarin content (2.69%) was observed with the low plant density in the second growing season (Table 6). Furthermore, there was significant interaction of plant density × year for silymarin yield. In the first growing season the greatest silymarin yield (63.3 kg ha^{-1}) was recorded for plant density B, while in the second growing season, the greatest silymarin yield (48.2 kg ha^{-1}) was recorded for plant density A.

Table 6. Effects of plant density and mepiquat chloride treatments on silymarin content (%) and silymarin yield (kg ha^{-1}) in 2015/2016 and 2017/2018.

Treatments	Silymarin Content (%)		Silymarin Yield (kg ha^{-1})	
	2015/2016	2017/2018	2015/2016	2017/2018
Plant density (D)				
Density A	2.83 a	2.69 a	54.2 b	48.2 a
Density B	2.87 a	2.26 b	63.3 a	33.0 b
$LSD_{5\%}$	-	0.42	4.77	10.74
Mepiquat chloride (MC)				
Control	2.73 a	2.39 a	55.6 a	41.9 a
MC75	2.84 a	2.35 a	58.8 a	36.1 a
MC100	2.81 a	2.69 a	58.3 a	43.0 a
MC112	3.01 a	2.46 a	62.2 a	41.5 a
$LSD_{5\%}$	-	-	-	-
	p-value from ANOVA			
D	0.137		0.285	
MC	0.642		0.687	
Year	0.006		<0.001	
D × MC	0.843		0.647	
D × Year	0.072		<0.001	
MC × Year	0.632		0.623	
D × MC × Year	0.943		0.898	

Density A: 28 plants m^{-2} and density B: 40 plants m^{-2}, MC 75: MC applied at the rate of 75 g a.i. ha^{-1}, MC 100: MC applied at the rate of 100 g a.i. ha^{-1}, and MC 112: MC applied twice, 2 weeks apart, at the rates of 37.5 g a.i. ha^{-1} and 75 g a.i. ha^{-1} for the first and second applications, respectively. Means followed by different letters within the same column indicate significant differences (for each factor) according to the Fisher's Least Significant Difference test ($p < 0.05$).

4. Discussion

4.1. Crop Parameters

Plant density had significant impacts on plant growth. The greatest height and rosette diameter were recorded with the lowest crop density of 28 plants m^{-2}. Similarly, the experiments of Andrzejewska et al. [14] that were conducted in Poland under different climate conditions revealed that the greatest values for plant height was recorded with the lowest density. On the contrary, Katar et al. [13], who conducted experiments in Ankara, Turkey, reported that the greatest values for plant height was observed with the higher plant density (25 × 10 cm). It is important to point out that our results showed that all crop parameters had greater values in 2016 than that in 2018; this is probably due to the greater amount of precipitation from March to May in 2016 (146.9 mm) compared with 2018 (61.9 mm). In addition, the precipitation in February of 2018 was higher (123.7 mm) in comparison to that in 2016 (14.3 mm), while the mean temperature in the same month was lower in 2018 (8.7 °C), in comparison to that of 2016 (12.0 °C). Ergo, these conditions retarded the growth of the milk thistle plants in the second growing season. This is supported by the results of the statistical analysis (Table 1), since a significant ($p < 0.001$) seasonal variation was observed for plant height. In addition, our results support earlier studies that found a strong influence of climate conditions on milk thistle growth [14,23]. It is also worth mentioning that the examined milk thistle genotype originating from central Greece produced high biomass production that ranged between 19,089 kg ha^{-1} to 25,440 kg ha^{-1}. This study indicated that milk thistle is a promising plant for energy production from biomass. Similarly, the experiments of Ledda et al. [23] that were conducted in Italy revealed that milk thistle is a suitable crop for energy production, while the biomass yield varied between 13,700 and 18,300 kg ha^{-1}.

Plant growth regulators, such as mepiquat chloride, prohexadione-Ca, and chlormequat chloride retarded plant growth (e.g., height reduction) in several crops, such as barley, cotton, sunflower, and wheat, due to the inhibition of gibberellin biosynthesis [19,24–27]. Our results revealed that mepiquat chloride (MC) had a significant effect on plant height, but it did not affect plant biomass. The largest height reduction was recorded with the MC 100 and MC 112 treatments. Furthermore, none of the MC treatments showed any phytotoxic effect on the milk thistle crop, while the MC application did not affect the relative chlorophyll content (SPAD values). In this regard, the application of MC in milk thistle can improve the harvest efficiency of this crop via the reduction of the seed dispersal during the harvest. The plant's height reduction varied between 8.39 to 14.83% in 2018 and was higher than that in 2016 (7.88–10.73%). To our knowledge, there are no reports regarding the effects of plant growth retardants on milk thistle height. Nevertheless, plant growth retardants are widely used in cotton crops. In a recent study, Choudhary et al. [26] observed that the application of plant growth regulators such as mepiquat chloride, mepiquat chloride plus cyclanilide, chlormequat chloride, and cyclanilide in cotton crops, reduced plant heights by 3.59 to 18.4%, while according to Tung et al. [27] plant heights were reduced by 9 to 40%, dependent on the application rates and the growing season.

4.2. Seed Yield and Components

For seed yield, a significant ($p < 0.001$) plant density × year interaction was observed. The highest seed yield was observed with the high plant density crops in 2016 and it was 15.19% higher in comparison to those of plant density A, while in 2018, the highest seed yield was observed from the lower plant density crops. Seed yield showed a positive and significant correlation with aboveground biomass ($r = 0.647$, $p < 0.01$) and rosette diameter ($r = 0.778$, $p < 0.001$). In a similar study, Katar et al. [13] reported that the largest seed yield was recorded from the highest plant density crops (25 × 10 cm). In contrast, Andrzejewska et al. [14] reported that seed yield was not affected by plant density and that the values varied between 550 and 1680 kg ha^{-1}. Moreover, the seed yield was higher in 2016 than in 2018, due to the higher precipitation during the flowering stage and seed development, as mentioned above. Similarly, Andrzejewska et al. [14] reported that the seed yield was dependent on weather conditions during the growing period, while Afshar et al. [28] observed that severe deficits of irrigation reduced the seed yield by 27%. Regarding the effects of plant density on the number of flower heads and 1000-seed weight, our results showed that the larger values were recorded with the low plant density only in the year with drought conditions during the period from March to May. The experiments of Afshar et al. [28] that were conducted in Iran under arid to semi-arid conditions revealed that seed weight was not significantly affected by water stress, while the number of flower heads per plant and number of seeds per head were sensitive to these conditions. Furthermore, mepiquat chloride did not affect seed yield and its components. In contrast, Geneva et al. [20] observed that prohexadione-Ca and mepiquat chloride in combination with fertilization increased both the number of flower heads and seed yield in milk thistle crops.

4.3. Oil Content and Yield

Milk thistle could be grown either as an oilseed or a biodiesel crop, since its seeds contain a considerable amount of oil [8,29,30]. In the present study, plant density and mepiquat chloride treatments did not impact the oil content that ranged between 24.2 and 27.3%. These values are comparable to those reported in the literature [8]. Our results also indicated that weather conditions played an important role in determining the oil content due to lower plant biomass in 2018, as mentioned above. This is supported by the results of the correlation analysis (Table 7), since oil content showed a positive and significant correlation with aboveground biomass ($r = 0.712$, $p < 0.01$) and rosette diameter ($r = 0.866$, $p < 0.001$). Moreover, calculated milk thistle mean value of oil yield was 478 kg ha^{-1}, lower than that reported for other oilseed crops. For example, Zheljazkov et al. [31] observed that the oil yield from sunflower growing in the USA ranged between 406 and 1166 kg ha^{-1}, while in another study conducted in Italy, Patanè et al. [32] reported that the oil yield of sunflower crop fluctuated between 790 and 2760 kg ha^{-1}.

Table 7. Pearson correlation coefficients[1] between the main crop parameters.

Crop Parameters	RD	SPAD	H	DW	HN	SW	SY	SilC	SilY	OC	OY
Rosette diameter (RD)	1	−0.015 ns	0.877 ***	0.604 *	0.721 **	−0.581 *	0.778 ***	0.822 ***	0.826 ***	0.866 ***	0.843 ***
Chlorophyll content (SPAD)	-	1	0.009 ns	−0.611 *	0.554 *	0.395 ns	−0.346 ns	−0.098 ns	−0.309 ns	−0.260 ns	−0.357 ns
Height (H)	-	-	1	0.525 *	0.746 ***	−0.647 **	0.772 ***	0.649 **	0.742 ***	0.729 **	0.804 ***
Dry weight (DW)	-	-	-	1	−0.030 ns	−0.780 ***	0.647 **	0.420 ns	0.600 *	0.712 **	0.710 **
Heads number (HN)	-	-	-	-	1	−0.201 ns	0.474 ns	0.588 *	0.506 *	0.460 ns	0.489 ns
1000-seed weight (SW)	-	-	-	-	-	1	−0.525 *	−0.290 ns	−0.467 ns	-0.653 **	−0.593 *
Seed yield (SY)	-	-	-	-	-	-	1	0.793 ***	0.969 ***	0.703 **	0.985 ***
Silymarin content (SilC)	-	-	-	-	-	-	-	1	0.913 ***	0.655 **	0.802 ***
Silymarin yield (SilY)	-	-	-	-	-	-	-	-	1	0.718 **	0.963 ***
Oil content (OC)	-	-	-	-	-	-	-	-	-	1	0.814 ***
Oil yield (Oϒ)	-	-	-	-	-	-	-	-	-	-	1

[1] r was calculated using the linear equation. $n = 16$. Significant at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ns: Not significant.

4.4. Silymarin Content and Yield

The silymarin content in this study varied between 2.26% and 3.01% of the dry seed weight. Our results showed that the highest plant density had a significant impact on silymarin content only during the second growing season. In particular, the lowest silymarin content was recorded with plant density B, while the silymarin content was lower overall in 2018, in comparison to 2016. The silymarin content in 2018 was 4.95% and 21.25% lower in comparison with 2016 for plant densities A and B, respectively. It is well known, that the silymarin content mainly depends on environmental conditions and genotype [8,14]. Thus, our results may be attributed to weather conditions during the period from March to May. The total precipitation during this period was lower in 2018 than that in 2016. Moreover, the precipitation in February of 2018 was higher in comparison to that in 2016, as mentioned above. These conditions in the second growing season retarded the plant growth especially in the plots with high plant density and consequently reduced the silymarin content. It is noteworthy to mention that the aboveground dry biomass in 2018 was 18.90% and 15.45% lower than that in 2016 for plant densities A and B, respectively. In a recent study Afshar et al. [16] observed that the concentration of silymarin increased by 4% and 17% with water stress conditions. The results of our study showed that the silymarin yield declined in the second growing season of 2018 due to the lower productivity associated with drought conditions. Silymarin yield varied between 33.0 and 63.3 kg ha^{-1} and showed a positive and significant correlation with seed yield (Table 7).

With regard to the effects of mepiquat chloride on silymarin accumulation, our results showed that the mepiquat chloride treatment had no impact on silymarin content or yield in 2016 or 2018. These results may be attributed to the fact that despite the reduction in plant height with MC treatments, the number of flower heads, 1000-seed weight, and chlorophyll content were not affected. In contrast, Geneva et al. [20] observed that the application of plant growth retardants, such as mepiquat chloride and prohexadione-Ca increased the silymarin yield in milk thistle, mainly due to an increase in seed production. Moreover, Stancheva et al. [21] reported that thidiazuron combined with foliar fertilizer increased silymarin content by approximately 10%.

Consequently, milk thistle could be a viable crop in most regions of southern Europe for seed production, while further research is needed to (a) establish the best cultivation practices (e.g., fertilization, rotation, and application of desiccants) for this crop and (b) to evaluate the productivity of several varieties and populations with different silymarin contents and flavonolignan compositions in regions with semi-arid conditions.

5. Conclusions

The results of the present study revealed that plant density had a significant impact on plant growth and physiology. The greatest values for aboveground biomass and seed yield were recorded with the highest plant density, while the highest values for plant height, rosette diameter, and chlorophyll content were recorded with the lowest plant density. Plant density had a significant impact on the silymarin content only in the year with drought conditions during the period from March to May.

In contrast, plant density did not affect the oil content of the seed. Regarding, the effects of mepiquat chloride (MC) on milk thistle growth and yield, our results showed that the MC application did not affect the majority of crop parameters such as plant biomass, relative chlorophyll content, silymarin and oil content, and silymarin production. Furthermore, mepiquat chloride reduced plant height, and the largest reductions were recorded with the MC 100 and MC 112 treatments. In addition, none of the MC treatments showed any phytotoxic effect on the milk thistle crop. Interestingly, in our report, several crop parameters including silymarin and oil content exhibited differences between the years, revealing the significant impact of climate conditions on milk thistle growth and production. The majority of the crop parameters had lower values in 2018 compared to that of 2016. Considering this, further research is needed to establish the best cultivation practices for milk thistle crop in regions with semi-arid conditions, while multi-year and multi-location studies are needed to shed light on the response of milk thistle to plant density.

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